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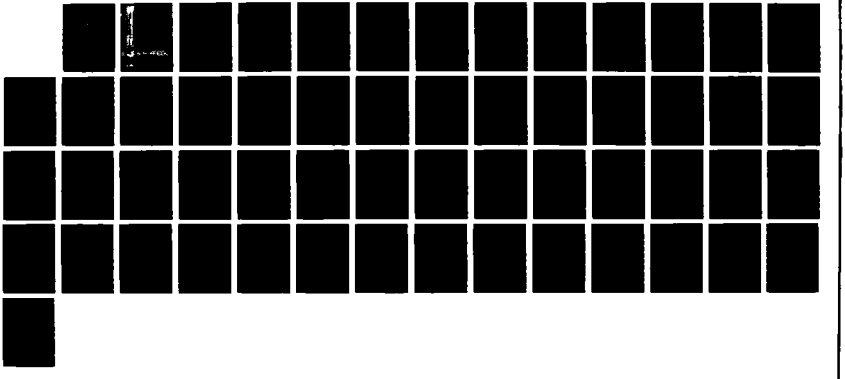

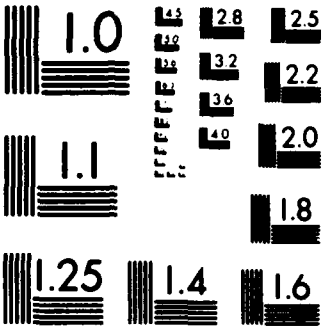

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THE MICROBIAL DEGRADATION OF TCE

J.H. WOLFRAM, J.T. CASE, R.D. ROGERS

AIR FORCE ENGINEERING AND
SERVICES CENTER
HQ AFESC/RDVW
TYNDALL AFB FL 32403-6001

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The objective of this research was to characterize naturally occurring microorganisms capable of degrading trichloroethylene (TCE). Microorganisms tolerant to TCE were isolated from an aeration tower at Wurtsmith AFB MI and from industrial sludges at Hill AFB UT. Isolates from the Wurtsmith samples exhibited the highest density of cells with TCE as the only apparent organic carbon source and were selected as the inoculum for degradation studies. However, the capability of selected microorganisms to degrade TCE was not substantiated in both gas chromatography analyses and samples innoculated in culture bottles. Experiments using radiolabeled carbon-14 to study assimilation suggest that TCE is metabolized into a polar form by the microorganism. Subsequent cultures grown in radiolabled TCE did not duplicate the previous results possibly because of contamination, selection of variants or loss of culture. The organism responsible for the apparent TCE degradation may belong in the genus Citrobacter.

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EXECUTIVE SUMMARY

A major portion of the investigation involved evaluation of trichloroethylene (TCE) biodegradation. Samples from environments containing TCE were enriched for organisms able to thrive under such conditions. Isolates from these enrichments were examined for TCE metabolism, using gas chromatography analysis. The data were not conclusive, as both the inoculated and control samples experienced loss of TCE from solution. Growth studies performed in the characterization tests showed that the bacteria adapted and could grow in the presence of TCE at concentrations up to 1000 µg/mL with no apparent effect on cell growth rate.

Trichloroethylene in solution was observed to decrease with time. Degradation of TCE could not be confirmed because of the variability seen in both the gas chromatography analyses and samples inoculated in the culture bottles. However, cells grew in the cultures and the experiments evaluating cosubstrates showed increased disappearance of TCE.

Experiment using radiolabeled TCE showed that a portion of the TCE was mineralized to $^{14}CO_2$. The bacterial cells produced during culturing were also examined for ^{14}C content. Carbon-14 was present in the cell material and a majority of the label could not be extracted from the cell with pentane, thus, indicating that the TCE was assimilated into the cell in a form other than TCE (metabolized form).

Two final experiments using radiolabeled ^{14}C did not duplicate the results observed in the previous radiolabel experiments. Changes in the bacteria or loss of organism viability are possible explanations for the differences. Isolates from the radiolabel cultures exhibiting TCE biodegradation have been preserved and are available for further study.

Physiological studies examined growth characteristics of one of the bacterial isolates (Wurtsmith 3). The isolate readily adapted to various concentrations of TCE ranging from 10-1000 µg/mL. The organism could grow in either aerobic or anaerobic conditions. Trichloroethylene

utilization however, was examined only under aerobic conditions. Optimal growth of this isolate occurred in buffered basal salts medium supplemented with 1 percent glucose. The organism did not grow on methanol, ethanol, or acetate but could utilize nitrogen in either organic or inorganic forms. The organism will grow on citrate if the pH is controlled. There was no evidence that the isolate could grow on other halogenated compounds similar to TCE.

Taxonomic investigations classify the Wurtsmith 3 isolate in the genus Citrobacter. The possibility still exists that TCE disappearance is a mixed culture phenomenon. Morphological examination of the media containing enrichment cultures contained various bacterial forms. A brief review of the literature did not reference the biodegradation capabilities of Citrobacter. These organisms are prevalent in both soil and water exposed to recalcitrant compounds such as TCE. The isolate has been adapted to grow in various concentrations of TCE through culture enrichment and degradation studies. Radiolabel experiments have shown partial TCE mineralization (~10-20 percent).

PREFACE

This report was prepared by the Biotechnology Group, Idaho National Engineering Laboratory, Idaho Falls, Idaho 83415, under Contract Number N-003-69, N-84-13, for the Air Force Engineering and Services Center, Engineering and Services Laboratory, (AFESC/RDVW), Tyndall Air Force Base, FL 32403-6001.

This report summarizes work done between 30 Apr 83 and 31 Jul 86. HQ AFESC/RDVS project manager was Maj Terry L. Stoddart.

This report has been reviewed by the Public Affairs Office (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nationals.

This technical report has been reviewed and is approved for publication.

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SECTION I

INTRODUCTION

A. OBJECTIVE

The specific objectives undertaken in the investigation include: (a) testing the hypothesis that trichloroethylene (TCE) is degraded through a microbial process, (b) identifying and classifying the organism(s) responsible for TCE degradation, and (c) providing a physiological, nutritional, and environmental characterization of the TCE isolates. Based on information provided by this study, additional work will be proposed to obtain a more TCE-tolerant organism (or organisms) and identify optimum conditions for TCE degradation.

B. BACKGROUND

TCE is an organic solvent used predominantly as a degreasing agent. The compound is not expected to persist in the environment because of its low water solubility, high-vapor pressure, and high atmospheric photodegradation rate. However, TCE is recalcitrant under conditions such as those associated with groundwater.

TCE-laden water can be treated using packed aeration columns. Removal of TCE from the water approaches 100 percent efficiency due to high TCE volatility (References 1, 2). Another removal method involves processing the water through a packed granulated carbon bed. This method is expensive.

Treatment of TCE-contaminated water at Wurtsmith Air Force Base, Michigan, employing an aeration tower, suggested biological treatment as a third possible method. Continued operation of the packed aeration tower to purge TCE from the water resulted in clogging of the tower with a biomass material, but TCE appeared to be removed. Organisms associated with the

biomass may have actively metabolized TCE, as this compound was the only available organic carbon source contained in the water.

The interaction between bacterial populations and environmental pollutants, such as TCE, which result in the partial or complete biodegradation of the pollutant is a promising research area. Studies have shown microbial degradation of many halogenated compounds (References 3-7).

A review of the literature presents conflicting evidence on the biodegradation potential of TCE. Lang has observed degradation of TCE under anaerobic condition, while studies by McCarty and Ritter showed no appreciable degradation (References 4, 8). Biodegradation screening studies of priority pollutants performed by the Environmental Protection Agency (EPA) in Cincinnati, Ohio, indicated that TCE can degrade aerobically. No specific information was found about the biodegradation rate of TCE in aquatic systems. TCE has been included in a list of synthetic organic compounds which should be degraded by microbiological treatment, provided suitable acclimatization can be achieved (Reference 9). Studies of compounds similar to TCE report that these low-molecular-weight chlorinated organics are not metabolized (Reference 9).

Conditions found in the Wurtsmith aeration tower provided the apparent ecological stress needed for development of a novel microbial community able to utilize TCE. Laboratory studies were instigated in an attempt to confirm that the biomass found in the tower degrades TCE.

C. SCOPE

In order to verify the microbial degradation of TCE, microoganisms were cultured under controlled environmental conditions while being exposed to TCE. Microbes which appeared to degrade TCE were isolated and their growth was characterized. While the work was bench-scale, the goal of the project was to develop a method which would lend itself to scale-up.

SECTION II

ISOLATION OF TCE-TOLERANT MICROORGANISM

A. SAMPLE SITE

A biomass sample was sent to the Idaho National Engineering Laboratory (INEL) by Headquarters, United States Air Force Engineering and Services Center (HQAFESC) in Spring 1983. The sample was collected from an aeration tower located at Wurtsmith Air Force Base. The sample consisted of the tower's packing material coated with a biomass film. Small plastic rings (~0.5 inches in diameter) were used for the packing. Air was forced up through the packing material, purging TCE from the water to the atmosphere. A film of biomass gradually accumulated on the rings, causing plugging of the tower. A second sample was sent to INEL in December 1983. The sample was composed of a biological filter system through which a large volume of the contaminated water had been processed and concentrated microorganisms contained in the water. Both samples were evaluated for TCE-tolerant microorganisms.

A review of the Hill Air Force Base (Ogden, Utah) Installation Restoration Program presented an additional opportunity to isolate potential TCE-tolerant organisms. Various base locations had been used as disposal sites for industrial chemicals over the previous 20 years, with TCE as the predominant chemical at a few of these locations. Water and sludge samples (described in Table 1) were obtained from the waste disposal sites. These samples were also screened for microorganisms able to degrade TCE.

B. CULTURE ENRICHMENT

Enrichment of the samples permitted the selection of microorganisms that could attack or degrade TCE and maintain themselves in the presence of the halogenated compound. The methods combined physical and chemical means designed to favor growth and multiplication of TCE-utilizing organisms. Three techniques were employed for enrichment: liquid culture, TCE

TABLE 1. HILL AIR FORCE BASE SAMPLES

Sample	Location	Comments
1	Industrial Wastewater Treatment Plant	Influent water sample (composite) water has been identified to contain large concentrations (ppm) of phenols, heavy metals, and organic solvents.
2	Industrial Wastewater Treatment Plant	Water sample obtained from the plant aeration basin.
Sediment	Industrial Wastewater Treatment Plant	Sludge sample from the plant's settling basin. Sample had very high water content.
3	Landfill No. 4 Discharge Seep	Water sample obtained from a small seep located at the perimeter of the landfill area. Water emerges at this location after migrating through the landfill. The waste disposal area was used for disposal of the industrial wastewater plant sludge and other waste chemicals.
4	Landfill No. 4 Discharge Seep	Replicate sample downstream of Sample No. 3
80-4	Landfill No. 4	Water sample from monitoring well located in the middle of the disposal area. This well drilled into a perched water zone (20 ft to 30 ft below surface) directly below the waste matrix.
80-24	Landfill No. 4	Water sample from monitoring well located at the perimeter of the disposal area. Perch water zone in this area represents water that has traveled through the waste matrix, i.e., water flows from sample Location 80-4 toward Location 80-24.
5	Chemical Disposal Pit #3	Water samples from monitoring wells. The disposal pit, operated from 1967 to 1975, received large quantities of sludge bottoms and other organics.
6	Chemical Disposal Pit #3	
7	Chemical Disposal Pit #39	

diffusion, and biphasic cultures. Enrichment media were prepared using the basal salts given in Table 2. The pH of the media was adjusted to give a final pH of 7.1. All media were heat-sterilized at 121°C for 15 minutes. TCE was added directly after the media had cooled.

TABLE 2. BASAL INORGANIC SALTS MEDIA[a]

Constituent	Concentration
Solution A	
$(NH_4)_2$ SO4	1.2 g
$CaCl_2 . 2H_2O$	0.1 g
Mg $SO_4 . 7 H_2O$	0.1 g
Fe SO_4	0.002 g
Yeast extract	0.01 g
Distilled water	1,000 mL
Solution B[b]	
K_2HPO_4	0.2 g
$KH_2 PO_4$	0.1 g
Distilled water	200 mL

a. Solutions A and B are sterilized separately, then aseptically combined prior to inoculation.

b. The buffered-basal salts media used a citrate (≤0.04 percent) - Na_2PO_4 solution in place of Solution B.

1. Liquid Enrichment Culture

The initial enrichment was undertaken using a liquid basal salts media containing TCE. An aliquot (1 mL) of each Air Force sample was added to a 250 mL Erlenmeyer flask containing 150 mL basal salts media. TCE was added to each flask in a concentration equivalent to 100 µg TCE/mL basal salts media. The enrichments were incubated for up to a month at room

temperature (20-25°C). The occurrence of growth in the cultures was indicated by turbidity and the appearance of flocs. Microscopic examination of the cultures showed dispersed growth of microorganisms. Cell plate counts on selected cultures showed cell concentrations of $\sim 10^6$ cells/mL.

2. TCE Diffusion Enrichment

In an effort to enrich for TCE organisms, cotton-stoppered flasks of basal salts broth were inoculated with 1 mL sample aliquots. The flasks were then incubated at room temperature (20-25°C) for 2 weeks in a closed chamber (desiccator) containing a beaker of TCE. The TCE diffused into the chamber atmosphere and the liquid broth, where it was available to microorganisms present in the sample. A control chamber without TCE was not used.

After incubation, streak plates were inoculated from each flask. Each plate consisted of a basal salts medium solidified with agarose (Sigma Chemical Company). The inoculated plates were placed in the chamber containing TCE and monitored for growth. Four of the culture plates exhibited colony growth. Small pinpoint colonies (<1 mm diameter) were observed under a dissecting microscope. Colonies were few (2-5 colonies per plate). Growth did not occur upon transfer to liquid enrichment broth. Gas chromatograph analysis of the basal salts broth (see Section III for method) incubated in the chamber showed that the TCE-laden atmosphere within the chamber saturated the liquid broth (and presumably the agarose plates) to a concentration approaching the solubility limit for TCE in water (1100 µg TCE/mL H_2O).

TCE present in the chamber atmosphere was also observed to cause deformation of the culture plates. During incubation, the plastic petri plates (Fisher Scientific Company, Pittsburgh, Pennsylvania) became very malleable. The plate lids had partially fused to the bottom plate section by the end of incubation. This apparent high TCE concentration present in

both the liquid broth and the chamber atmosphere could have had a toxic effect on any organisms present in the enrichment cultures.

3. Biphasic Mass Culture

TCE had to be readily available to the microorganism(s) to enable culture enrichment. The physical properties of TCE presented somewhat more difficult means to achieve enrichment. These physical properties are listed in Table 3 (References 9, 10). At room temperature, TCE is a clear, colorless, noncorrosive, nonflammable liquid. The high TCE vapor pressure results in the disappearance of the compound from solution via evaporation. The concentration of TCE in water has been observed to decrease significantly within several hours with agitation and exposure to the atmosphere (References 5, 11, 12). A 50 percent loss by evaporation occurs in less than 30 minutes under these conditions (Reference 12).

TABLE 3. PHYSICAL PROPERTIES OF TRICHLOROETHYLENE

Molecular weight	131.39
Melting point	-73°C
Boiling point at 760 torr	87°C
Vapor pressure at 20°C	57.9 torr
Solubility in water at 20°C	1100 mg/L
Log octanol/water partition coefficient	2.29

The rapid disappearance caused by evaporation and low solubility in water makes it difficult to maintain TCE in an environment conducive to biodegradation. An enrichment technique employed to minimize these effects was biphasic mass culture. The method essentially represents a form of dialysis/diffusion culture. The biphasic mass culture consists simply of a thick layer of solidified (using agarose) enrichment medium, overlaid with a

thin layer of liquid broth. Before cooling, TCE is added to the thick agarose layer. The TCE contained in the solidified layer can readily diffuse into the liquid overlay. The interface between the two physically different phases acts as a dialysis membrane, allowing TCE to continuously diffuse into the liquid broth. The design approximates a "continuous" culture, i.e., chemostat system. The method allows for prolonging the exponential growth phase, resulting in a dense concentration of cells (Reference 13). Therefore, a more constant environment can be provided to cells grown in mass culture. In addition, the effects of evaporation and solubility can be minimized by the equilibrium that results from the steady diffusion into the liquid overlay. Figure 1 shows TCE diffusion into the liquid overlay as a function of time. It was found that the concentration of TCE in the liquid overlay depends on the thickness of the agarose base layer, the liquid overlay thickness, and the amount of TCE added to the agarose layer. The investigator can adjust these parameters to obtain optimized growth conditions. Using this method, growth was observed in both the Wurtsmith Air Base samples, and in Hill Air Base samples. The biphasic growth study results are described in Table 4.

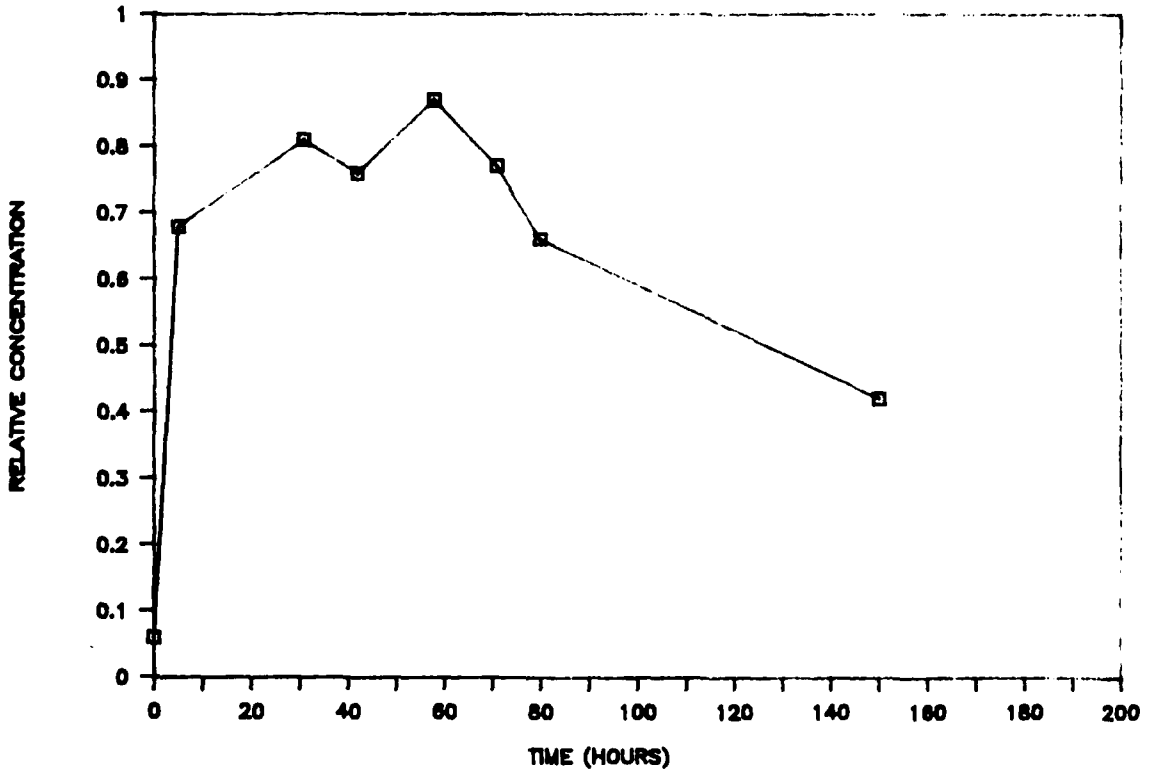

Figure 1. Biphasic Mass Culture Substrate Diffusion

TABLE 4. ENRICHMENT CULTURE GROWTH STUDIES[a]

Sample Identification[b]	Observation	Predominant Microflora
1	Growth	Mixture, gram-positive rods, rods, short plump and long rods.
2	No growth	
3	Growth	Gram-positive rods.
4	Growth	Mixture, gram positive, rods some gram-positive branching rods.
5	Growth	Gram-positive rods.
6	Growth	Gram-positive elongated rods.
7	Growth	Gram-positive rods.
80-4	Growth	Gram-positive rods.
80-24	Growth	Gram-positive short plump (rounded ends) rods.
Sediment	Growth	Mixture, gram-positive rods, filamentous.
Air Force Isolates	Growth	Gram-positive elongated rods, some branching.

a. Biphasic basal salts culture containing 180 µg/mL TCE.

b. Table 3 describes each sample.

SECTION III

EVALUATION OF TCE BIODEGRADATION

A. INITIAL STUDIES

Halogenated hydrocarbons such as TCE in water can be readily detected through gas chromatographic methods. The initial experimental studies were designed to measure disappearance of TCE in a liquid basal salts culture, inoculated with a sample of the enriched Air Force biomass. The analytical technique involved direct injection of a culture sample liquid broth aliquot into the gas chromatograph for TCE analysis. Direct injection of the sample is simple and fast and minimizes the likelihood of artifacts which can occur with sample manipulation. A Hewlett-Packard Model 5710A gas chromatograph with a flame ionization detector (FID) was used for the analysis. The column is 8 feet by 1/8 inches outer diameter, stainless steel packed with 60/80 Carbopack® B/1 percent SP-1000 (Supelco, Inc., Bellefonte, Pennsylvania). The initial analysis used the temperature program oven (45-210°C at 8°C/min) with nitrogen as the carrier gas.

The experiments were carried out using the previously described sterile liquid basal salts medium. Sterile serum bottles (160 mL volume) filled with 60 mL of media and 1 mL of a bacteria inoculum enriched from the original Wurtsmith biomass sample were used in the study. Controls were identical to the treatments except they were not inoculated. TCE was aseptically added to each bottle in concentrations ranging from 50-400 µg/mL. All culture bottles were tested in duplicate. One-microliter samples were withdrawn over time from each bottle using a 1 µL syringe (Hamilton Series 7000, Reno, Nevada) and injected into the gas chromatograph for analysis. Results are shown in Table 5.

These data show a decrease in TCE concentration with time in both treated and control bottles. The losses occurred within the first week of sample incubation. Additional analyses of control samples indicate that TCE

TABLE 5. REMOVAL OF TRICHLOROETHYLENE BY BATCH CULTURE

Sample (TCE added to each bottle, µg/mL)	TCE in broth at 0 time (µg/mL)	TCE in broth after 7d (µg/mL)
Inoculated Bottles		
50	42	7
100	50	N.D.[a]
200	135	23
400	269	93
Control Bottles		
50	35	9
100	56	20
200	125	44
400	332	95

a. N.D.--not detected

is completely removed from both control and seeded broth within a month. The main mechanisms for the TCE loss appear to be evaporation and diffusion of the chlorinated compound through the pierced seals. To alleviate this problem Teflon®-lined Mininert® valves (Precision Sampling Corp.) were acquired for sealing the culture bottles. The valves possess a double-septum (Teflon®-lined) seal which allows withdrawal of the sample without loss or exposure. Evaporation of TCE is assumed to be confined to the sample headspace above the culture broth when the valves are used to seal the culture bottles.

A second experiment was initiated examining TCE disappearance in culture bottles sealed with the Mininert® valves. TCE was added to all the samples in a concentration equivalent to 175 µg/mL in 100 mL of broth. Samples were tested in triplicate. Seeded cultures were inoculated from enriched Wurtsmith cultures. The bottles were incubated at room

temperature and all the samples were sealed using the Mininert® valves. Every 1-2 weeks, each bottle was assayed for TCE by the direct. sample injection gas chromatographic procedure.

Figure 2 shows the change in TCE concentration over a 6-week period in sample bottles with and without the inoculum. As can be seen, a peak in TCE concentration occured in both the inoculated and control samples within the first week of incubation. This rise of TCE levels in the broth is attributed to the compound's low solubility in water. TCE slowly solubilized into the liquid until an equilibrium is reached. After this peak concentration was observed, both the control and seeded bottles exhibited a decrease with time in the amount of TCE contained in the liquid phase of the cultures. Difference observed between the seeded and control cultures include a lower equilibrium (peak) concentration of TCE in the seeded bottles and a 20 percent greater loss of TCE from the seeded culture broth at the end of the experiment. This loss was not statistically significant because of the small number of replicates examined.

The experimental data indicate that a large fraction of TCE is removed from the broth by abiotic processes. Duplication of the experiment showed similar results. Figure 3 shows the TCE loss in control and seeded cultures in the repeat tests. Again, the seeded bottles exhibited a more rapid decrease, as compared to controls. Unfortunately, sample analysis had to be discontinued after the first week because of contaminated controls. The cell population measured in the sample bottles was 10^6 cells/mL. Thus, the microbial growth which could be supported by the culture medium was low. In an effort to increase biomass production, additional experiments were carried out using a second carbon source in conjunction with TCE. It was intended that a cosubstrate could provide the organic nutrient necessary to support increased production of the biodegrading microorganisms. Cometabolism of TCE can also be evaluated. Glucose and citrate were added (separately) to the growth medium with TCE at a concentration of 1 µg/100 mL. These two compounds were chosen because they are relatively inexpensive and common metabolites for aerobes. Sample bottles were inoculated (except controls) with the Wurtsmith isolates and the concentration of TCE was analyzed at selected time intervals.

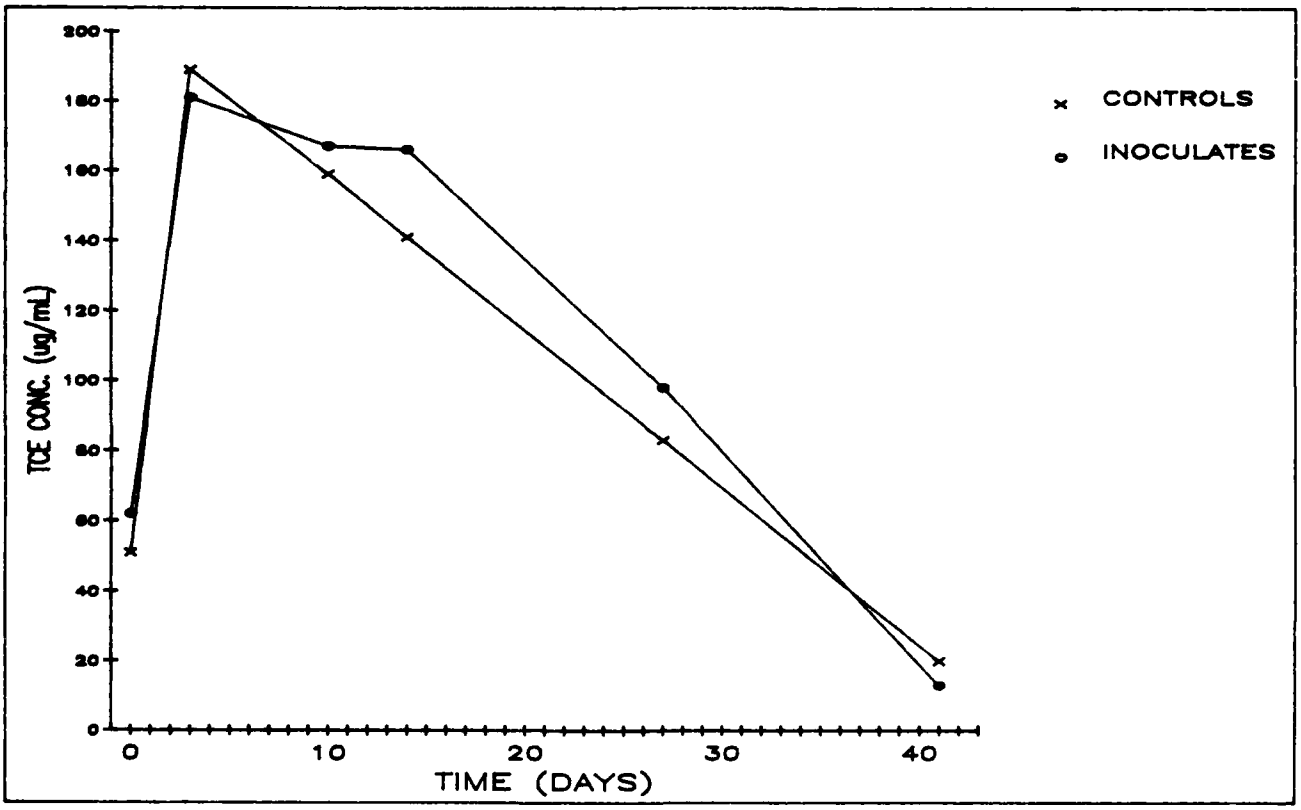

Figure 2. TCE Concentration vs Time in Batch Cultures

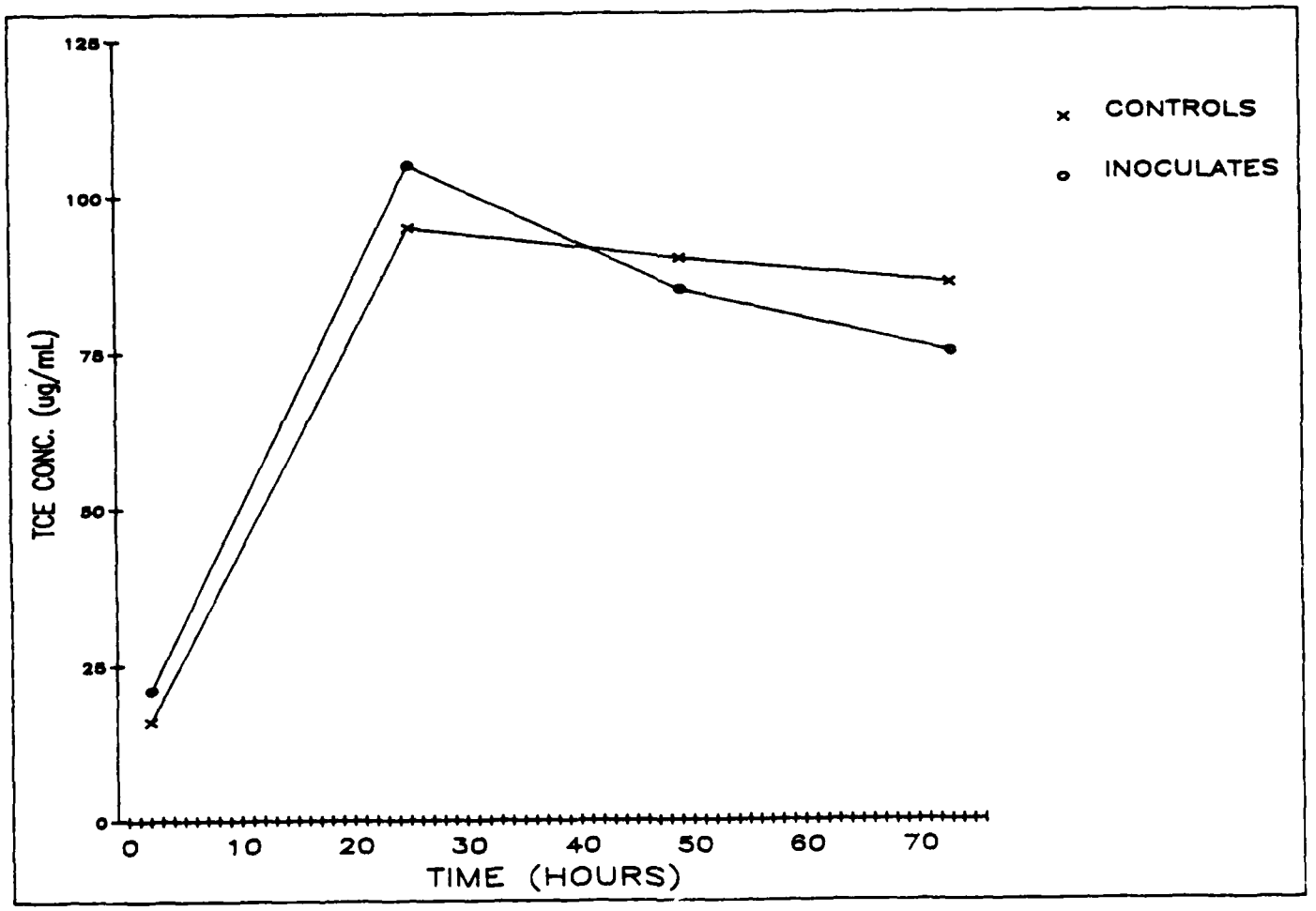

Figure 3. TCE Disappearance from Batch Culture, Second Trial

The fate of TCE under cosubstrate conditions can be seen in Figure 4. In the presence of glucose, TCE concentration had a 36 percent greater decrease with time relative to the controls. The addition of citrate did not favor increased TCE degradation. It became apparent during nutritional studies (see Section IV) that the buffering capacity of the mineral salts was inadequate when citrate was added to the broth. The pH of the mineral salts medium decreased to 4 in a 1 percent citrate (w/v) basal salts culture broth. This low pH severely limited microbial growth.

B. RADIOLABEL EXPERIMENTS

The cosubstrate microcosm studies utilizing gas chromatography with FID indicated that some of the enriched cultures biodegraded TCE. To further define the fate of TCE, radiolabel experiments were begun using Carbon-14 (^{14}C) labeled TCE (New England Nuclear Corp., Boston, Massachusetts). Specifications of the label are given in Table 6.

TABLE 6. RABIOLABELED TRICHLORETHYLENE SPECIFICATIONS

Activity:	250 microcuries
Form:	Trichloroethylene (≥98 percent purity) contained in 20 mL distilled water sealed in glass ampule
Activity Concentration:	8.5 millicuries/millimole
Label:	Carbon-14 on C_2 atom

The radiolabel and physical purity of the labeled TCE was determined on freshly opened vials of the labeled solutions. First, a sample of the radiolabel was analyzed with a liquid scintillation detector to quantify ^{14}C activity. Then, another sample was extracted with pentane to separate the TCE from its water carrier. This was accomplished by injecting 2 mL of the aqueous TCE through a septum (Teflon®-faced rubber) into a 5 mL microreaction vial contained in 1 mL of pentane. The contents were then mixed with a vortex mixer for 1 minute and then set aside until the contents separated

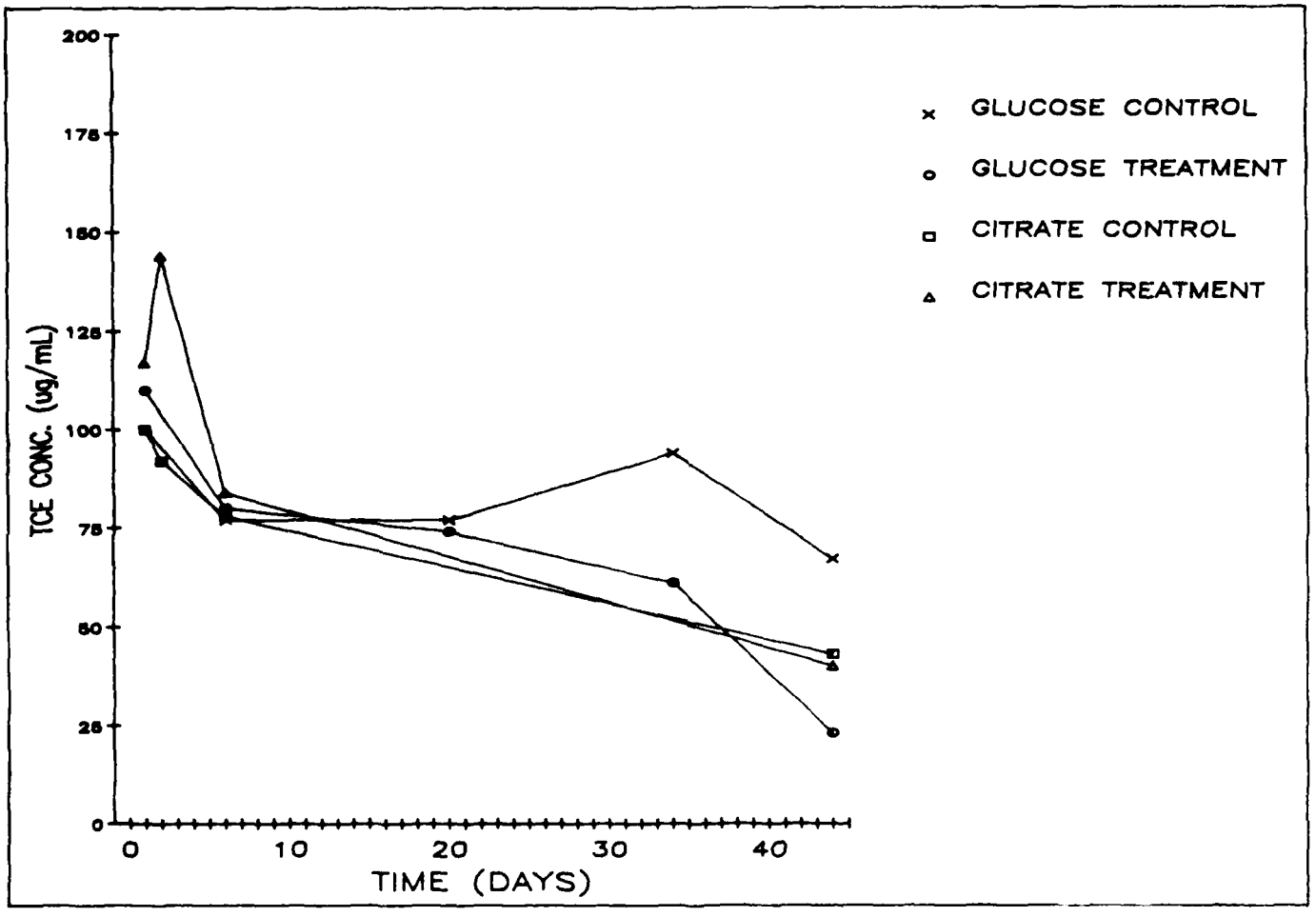

Figure 4. TCE Concentration in the Presence of Cosubstrate

into aqueous and pentane layers. Samples of the separated layers were then analyzed by liquid scintillation to determine the quantities of ^{14}C in each. In addition, the pentane (1 µL) was analyzed by a gas chromatograph with an electron capture detector (ECD) to verify that TCE was the only halogenated compound present in the radiolabeled solution. A summary of the TCE extraction efficiency liquid scintillation measurements is provided in Table 7.

TABLE 7. RABIOLABELED 14_C-TCE EXTRACTION EFFICIENCY

Sample	^{14}C 10^7 dpm/mL
Vendor-supplied ^{14}C trichloroethylene solution	1.31
Extractable ^{14}C (pentane layer)	1.05
Extraction efficiency	80.2 percent

To check the efficiency of extracting ^{14}C TCE from water, the same procedure was employed with an unlabeled standard of TCE in water which was prepared in the laboratory. The extraction efficiency for this mixture (as determined by ECD), was similar to that of the ^{14}C TCE (80 percent). These values appeared low when compared with an expected efficiency value of near 100 percent. However, because the values for TCE were obtained from two separate sources and analyzed by two different methods (either liquid scintillation or ECD), there was an implied confirmation that the method, rather than the quantity of TCE, was responsible. Figure 5 is the quality assurance report supplied by the manufacturer of the radiolabeled TCE, while Figure 6 shows our laboratory's ECD tracing for the labeled solution.

After this initial examination of the labeled TCE, experiments were planned to examine the carbon flow through a TCE-metabolizing culture. It was decided three compartments would be analyzed by liquid scintillation for ^{14}C activity: the culture medium, the cellular material, and the gas

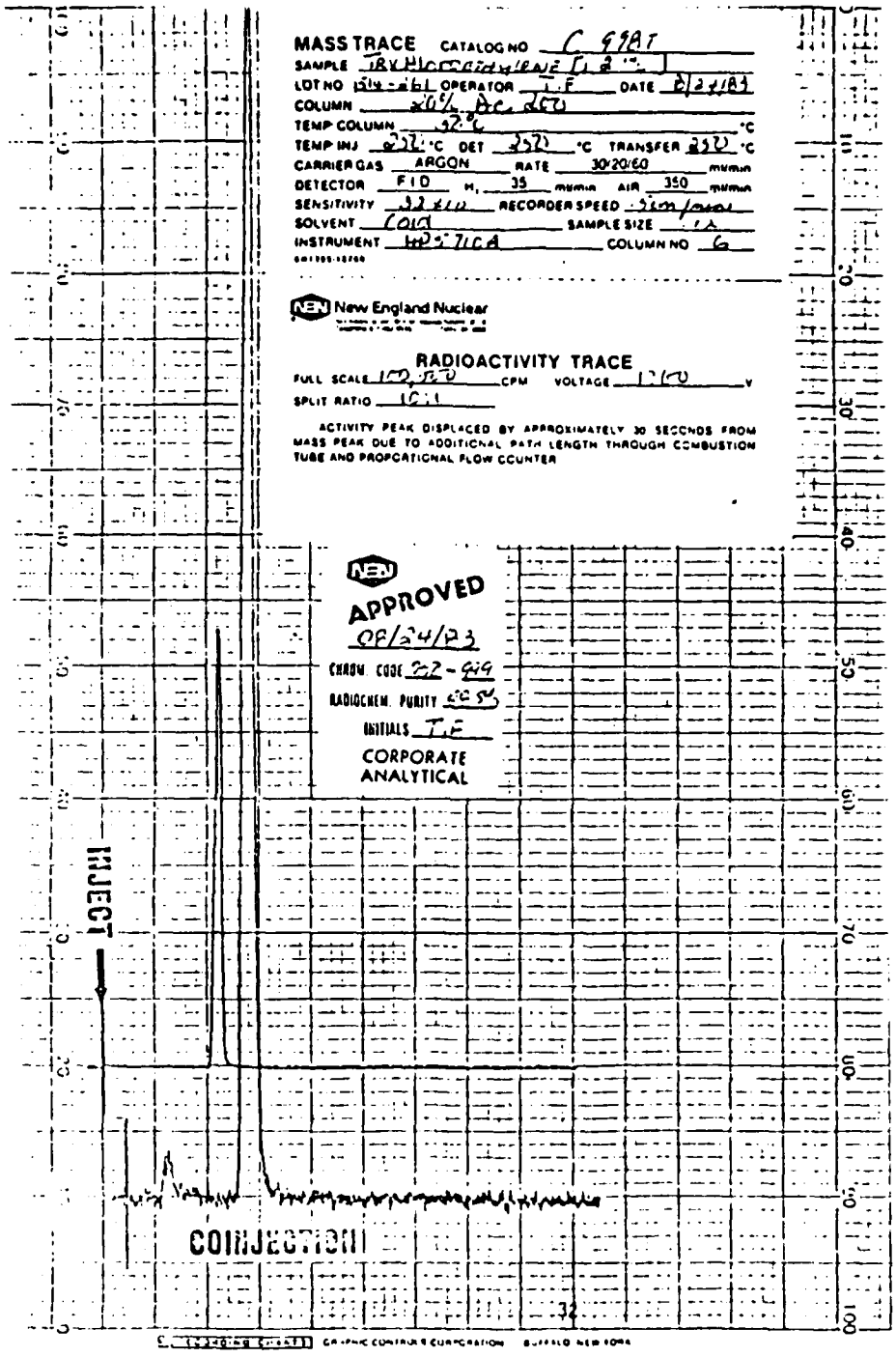

Figure 5. Radiolabeled TCE Quality Assurance Data

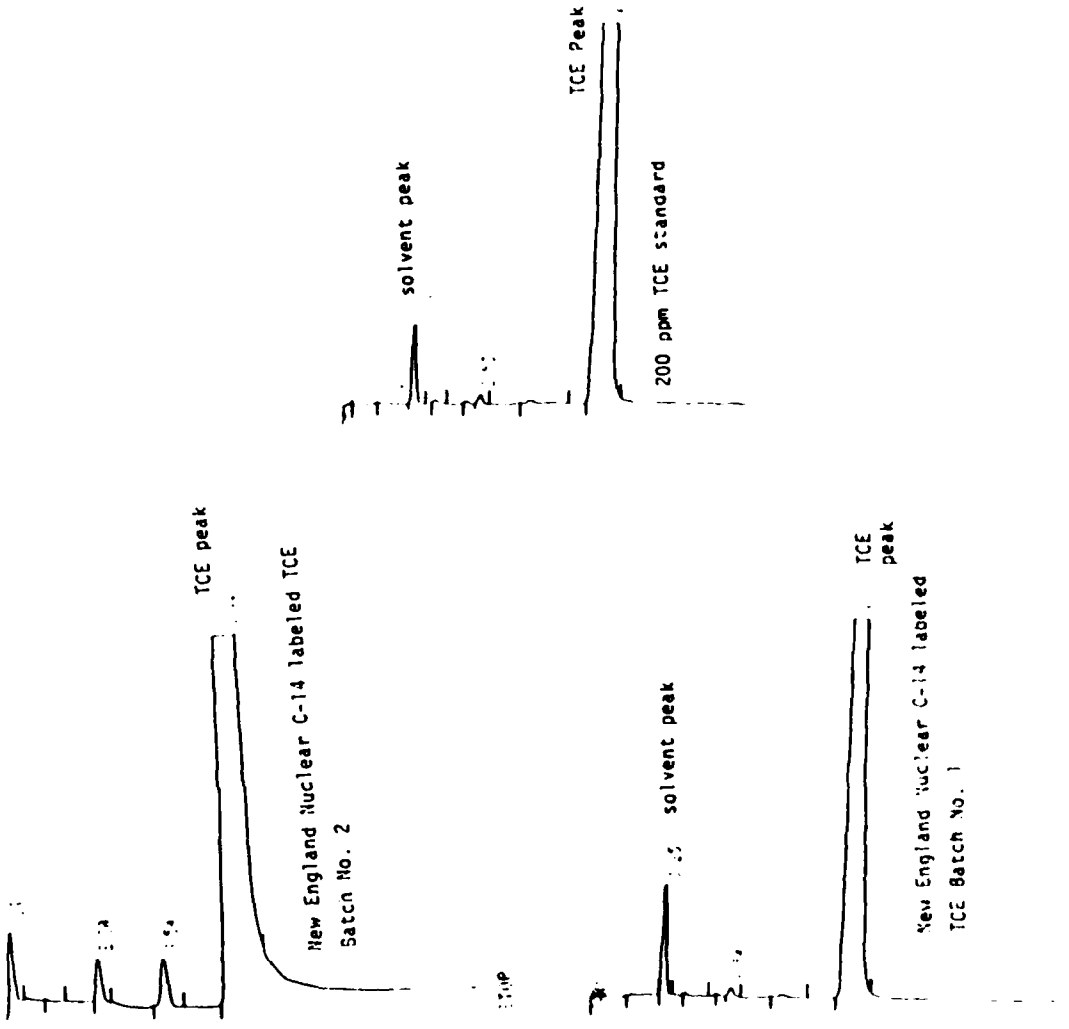

Figure 6. Radiolabeled Chromatogram

phase for CO_2 production. Screening of culture strains was also to be performed in order to select those capable of utilizing TCE.

The concentration of TCE to which cultures were exposed was 100 µg TCE/mL media. Labeled TCE obtained from the NEN stock was used in a ratio of 1:25 with the stable TCE. Samples intended for liquid scintillation analysis were prepared in Scintiverse II® (Fisher Scientific Company, Pittsburgh, Pennsylvania) scintillation fluid. Carbon-14 activity in the samples was determined by counting the samples in a Packard Model 3385 TRI-CARB® or a Beckman LS-150 liquid scintillation spectrometer. Quench correction and counting efficiency were determined using the external standard channels ratio method (References 14, 15). Carbon-14 labeled carbon dioxide gas was trapped using the inorganic base potassium hydroxide (KOH) (Reference 16). Ethanolamine was evaluated as a $^{14}CO_2$ trap but was discarded because of the compound's affinity for volatile TCE. The TCE dissolved in the ethanolamine and could not be separated with solvent extraction. This problem was not encountered when 1N KOH was used as a $^{14}CO_2$ trapping agent because carbon-14 labeled TCE could be extracted from the KOH with pentane. Any label remaining in the KOH after extraction was assumed to be a nonextractable polar compound. It is known that chemiluminescence can result from a liquid scintillation cocktail with an induced alkaline pH (a phenomenon caused by the OH^- rather than either of the cations Na^+ or K^+) interacting with peroxides (p. 28, Beckman Instrument Co. Bulletin 7397A). Therefore, duplicate mixtures of scintillation cocktail (18 mL) and either 1N KOH or NaOH (2 mL) were analyzed for chemiluminescence. The analysis was performed within an hour of mixing with counting occurring in the tritium, carbon-14 and phosphorus-32, channels. Initial counts averaged 2429 cpm and 1633 cpm, respectively, for KOH and NaOH in the tritium channel, 31 cpm and 24 cpm for the carbon-14 channel, and 65 cpm and 32 cpm with the phosphorus channel. A water (2 mL) blank in scintillation cocktail averaged 142 cpm, 20 cpm, and 32 cpm in the same order of channels. These data showed that chemiluminescence was not expected to be a significant cause for error in those experiments involving the use of KOH when counting carbon-14. As an

added precaution, counting data used for tabulating final results were obtained from samples which were dark-adapted overnight.

The final radiolabel experimental series was initiated to screen the enriched culture samples for microorganisms able to degrade TCE. The experiments were conducted in 250 mL Erlenmeyer flasks containing a center well in which 5 mL of 1N KOH was placed. Sterile basal salts broth (100 mL) was amended within each flask with sufficient unlabeled (neat) and labeled (from the aqueous C-14 TCE stock solution) TCE to provide a concentration of TCE equivalent to 100 µg/mL broth (four parts labeled TCE to 96 parts unlabeled TCE). Flasks and their contents were then incubated at 25°C for 24 hours to allow the unlabeled and labeled TCE to reach equilibrium. An aliquot of cells from each enriched culture was added to all flasks except controls. A sample of the media from each flask was taken at the start of the growth period to determine initial TCE concentration. The cultures were incubated for 2 weeks at 25°C. This and subsequent experiments were designed to determine carbon partitioning within a culture. Therefore, at the end of incubation, each culture flask was sampled to determine ^{14}C activity remaining in the liquid media, in the cellular material, and as $^{14}CO_2$ in the trapping agent. These analyses were performed by liquid scintillation on 2 mL of the broth, 4 mL of the center well KOH and a 2 mL solution containing the flask cell contents. The cells were prepared by centrifuging the broth culture to obtain cell pellets which were later washed three times with a sterile buffer solution (via resuspension and centrifugation).

The screened samples included both Wurtsmith and Hill Air Force Base cultures. These samples had exhibited growth in the TCE biphasic mass culture enrichment studies. All the sample flasks, including the controls, contained ^{14}C in the $^{14}CO_2$ trap. The ^{14}C measured in the control flask $^{14}CO_2$ traps indicated that TCE had evaporated from the culture broth and a portion had diffused into the potassium hydroxide trapping solution. A 31-51 percent decrease in broth ^{14}C concentration relative to the controls was observed in three of the samples. Two of these samples

also contained more (by a factor of 10) ^{14}C in the cellular material than was present in the other samples. These two cultures, isolated from the original Wurtsmith biomass sample, had previously exhibited apparent TCE biodegradation when grown in a cosubstrate (glucose and TCE) growth medium.

The screening experiment showed that some of the cultures do uptake TCE from the growth medium as was evidenced by the redistribution of ^{14}C to KOH and the biomass. However, the results did not differentiate the form of ^{14}C present in the broth, the cells, or in the $^{14}CO_2$ trap. To verify the initial finding, a second radiolabel experiment was performed with two promising isolates to examine the three compartments in detail. Experimental conditions were the same as in the screening studies except that all cultures were amended with 1 percent glucose (w/v). Previous results (see Figure 4) indicated that TCE loss increased in the presence of the cosubstrate glucose. The isolates used were those which had exhibited the greatest TCE utilization in the gas chromatography and radiolabel screening experiments. Both the isolates were enriched from the Wurtsmith Air Base samples and were given the designation of Wurtsmith Isolates 3 and 4. In this experiment, the additional technique of using pentane to extract the compartments of the culture was used. Testing had shown that TCE or other nonpolar organic compounds present in the sample can be concentrated in a pentane extraction layer (Reference 18). At the end of incubation, ^{14}C activity measured in the KOH CO_2 trapping solution, the broth, and the cellular material. All of these samples were extracted with pentane. Extraction was accomplished by adding 2 mL of pentane to a 5 mL reaction vessel containing 3 mL of the sample to be extracted. The reaction vessel was then mixed by shaking a vortex mixer for 3 minutes. Pentane and water layers were then separated and analyzed for ^{14}C activity. It was assumed that ^{14}C measured in the pentane fraction would be in the form of TCE or other extractable organic and that any ^{14}C remaining in the aqueous fraction (after extraction) would be assumed to be metabolized TCE. Thus, the TCE carbon could be in the form of $^{14}CO_2$ (contained in the trapping solution), a metabolite (contained in the broth), or as assimilated carbon (if found in the cellular material).

The experiment results in Table 8 show that with both isolates ^{14}C activity in the broth decreased by 30-42 percent. In addition, after incubation only $\leq$3 percent of the ^{14}C activity could be extracted from the broth. The same situation was observed in both the KOH trapping solution and the cellular material; i.e., both components assimilated ^{14}C activity but only a small fraction was extractable with pentane. These experimental data indicate that some partial and complete mineralization of TCE could

TABLE 8. ASSESSMENT OF OF BIODEGRADATION RADIOLABELED PORTION OF TOTAL TCE FOR INITIAL RADIOLABELED TCE EXPERIMENT

Samples	Wurtsmith Isolate 3 (10^{-8} Ci/mL)	Wurtsmith Isolate 4 (10^{-8} Ci/mL)
Initial C-14 TCE broth concentration	1.0	1.2
Final C-14 TCE broth concentration	0.70	0.70
Final C-14 TCE broth pentane layer concentration	0.02	0.02
Percent extractable broth C-14	3.0%	2.7%
CO_2 trapping solution C-14 concentration	2.4	0.90
CO_2 trapping solution pentane layer C-14 concentration	0.07	0.07
Percent extractable CO_2 trapping solution C-14	2.8%	7.8%
Assimilated cellular C-14	0.40	1.0
Assimilated extractable cellular C-14	0.003	0.03
Percent extractable assimilated cellular C-14	0.7%	3%

have been accomplished by these cultures. Carbon-14 activity was measured in the culture broth, the CO_2 trapping solution, and the cellular material in a form other than TCE.

Morphological examination of Wurtsmith isolates 3 and 4 indicated that each consisted of mixed organisms that could have been related strains. All isolates were gram-negative motile rods. Therefore, when the experiment was repeated a second time, only Wurtsmith 3 was used. The same experimental protocol used in the previous radiolabel studies was followed, with an emphasis on examining TCE behavior in the controls. Table 9 presents a distribution of the radioactivity observed at the end of the study. Cultures were initially dosed with labeled and unlabeled TCE (100 µg/mL) and incubated for 1 week. The three culture compartments (broth, CO_2 trapping solution, and the cellular material) were examined for ^{14}C content. Each compartment was extracted with pentane to remove any organic nonpolar soluble radiolabeled compounds, i.e., TCE or partially degraded nonpolar metabolites. The data show a large decrease (72 percent) in the culture ^{14}C activity (both control and inoculates). TCE was again observed to diffuse into the KOH CO_2 trapping solution. However, extraction data indicate that 8 percent of the added TCE could have been converted to $^{14}CO_2$ during incubation. A smaller amount (1.1 percent) of the label was contained in the cellular material after washing cells a minimum of three times. Nearly 90 percent of this cellular fraction remained with the cell after extraction with pentane. Only 6 percent of the radioactivity remained in the inoculated broth aqueous extraction. This represents a much smaller ^{14}C concentration than was observed in the earlier radiolabel experiment. Both the control and the inoculated cultures exhibited large TCE losses. Control data from each investigation indicate that TCE does not remain in the culture, i.e., physical losses occur. It is difficult to differentiate between biological and physical losses of TCE. However, cellular uptake of the radiolabel and possible $^{14}CO_2$ production were indications that TCE was being biodegraded.

TABLE 9. DISTRIBUTION OF RADIOACTIVITY IN ^{14}C-LABELED TCE CULTURE

	Radioactivity[a]			
	Inoculum		Control	
Source	10^5 dpm	% of input	10^5 dpm	% of input
TCE in solution	13.0	27.7	11.0	27.6
Potassium hydroxide solution	3.4	8.0	1.7	4.0
Assimilated in cellular material	0.52	1.1	--	--

a. All measurements made after 1 week incubation.

To further define the physical behavior of TCE in the cultures, radiolabeled TCE was added to a series of test flasks containing sterile media and the TCE dispersal was measured over time. These controls were divided into the following four groups: (a) sterile basal salts media with distilled water in the CO_2 trap, (b) sterile basal salts media with KOH in the CO_2 trap, (c) sterile distilled water media with distilled water in the CO_2 trap, and (d) sterile distilled water media with potassium hydroxide in the CO_2 trap. Basal salts and distilled water were employed as the culture media to determine if the salts solution interfered with TCE extraction. Labeled and unlabeled TCE was added to each flask in a concentration equivalent to 100 μg/mL. Each flask was sealed using Teflon® Mininert® valves and glass joint stoppers. The flasks were specially made so that samples of the culture media and CO_2 trap solution could be withdrawn without opening the flask to the atmosphere. All the flasks were incubated at 25°C and samples were withdrawn over an 8-day period.

The data for this experiment (Tables 10 and 11) were converted from dpm into μg of total TCE. The concentration of TCE in both the basal salts and distilled water media showed a large decrease (37 percent) in the

TABLE 10. PHYSICAL BEHAVIOR OF TCE IN FLASK SOLUTIONS

Time	Contents of Flask/Contents of Trap			
	H_2O/H_2O	H_2O/KOH	BS[a]/H_2O	BS/KOH
	µg TCE/100mL			
0	7300 ± 2300	10000 ± 1700	8400 ± 1100	9500 ± 1900
24	5200 ± 100	5700[b]	6100 ± 500	5400 ± 400
96	5000 ± 200	5400[b]	5000 ± 300	5300 ± 600
192	4500 ± 1000	5900 ± 1200	4800 ± 400	5300 ± 1100

a. Basal Salts media.

b. Duplicate not sampled.

TABLE 11. OCCURRENCE OF TCE IN SOLUTIONS CONTAINED IN CO_2 TRAPS

Time	Contents of Flask/Contents of Trap			
	H_2O/H_2O	BS[a]/H_2O	H_2O/KOH	BS/KOH
	µgTCE/20mL			
0	210 ± 40	160 ± 30	120 ± 10	120 ± 20
24	800 ± 10	830 ± 130	390 ± 30	370 ± 30
96	550 ± 30	750 ± 60	590 ± 40	490 ± 60
192	450 ± 100	650 ± 70	790 ± 70	670 ± 150

a. Basal Salts media.

first 24 hours of incubation (Table 10). After this initial loss of TCE from solution, the compound diffused from both solutions at a much slower rate. An additional 14 percent decrease in TCE activity was observed in the distilled water between 24 and 192 hours. The basal salts experienced an additional 9 percent loss in TCE over the same period.

Differences were observed in the amount of TCE which diffuses into the CO_2 trap solutions (Table 11). Both solutions, distilled water and KOH, contained TCE. TCE diffuses into distilled water at an accelerated rate compared to diffusion into the KOH. The TCE activity in water reached a maximum level within the first 24 hours of incubation, then showed a gradual diffusion out of the solution. The TCE concentration present in the KOH was still increasing at the end of the study (196 hours) while there was a decrease with time in the distilled water. Extraction of the samples with pentane indicated that the basal salts or KOH solutions did not interfere since an extraction efficiency of 80 percent was observed for both salt solutions (basal salts and KOH) and in the distilled water samples.

This experiment showed that, in a sealed system, TCE will move toward an equilibrium with the surrounding atmosphere and other isolated solutions. Most of the TCE liberated from the broth could not be accounted for and was assumed to be in the unsampled flask atmosphere. Because of this, it was not possible to calculate a mass balance or to assume that ^{14}C activity in the KOH solution confirmed the production of $^{14}CO_2$. However, after it was found that TCE could be removed from the KOH by extracting with pentane, there was some evidence that the remaining nonextractable compound seen in the KOH solutions of previous experiments could have been $^{14}CO_2$.

To verify CO_2 production, a method was devised to flush the culture flasks' atmosphere with five volumes of air, through an external pentane and 1N KOH trap (10 mL each). In this way, TCE would be removed first by the pentane while $^{14}CO_2$ would be contained in the KOH. Figure 7 is a schematic of the method. After flushing, 1 mL of 2 N HCL would be added to 1 mL of the KOH samples. This facilitated the removal of CO_2 from the solution. The amount of labeled $^{14}CO_2$ present in the KOH could then be estimated by comparing the ^{14}C activity contained in the samples before and after acidification. The difference between the two ^{14}C measurements would then be attributed to the loss of $^{14}CO_2$.

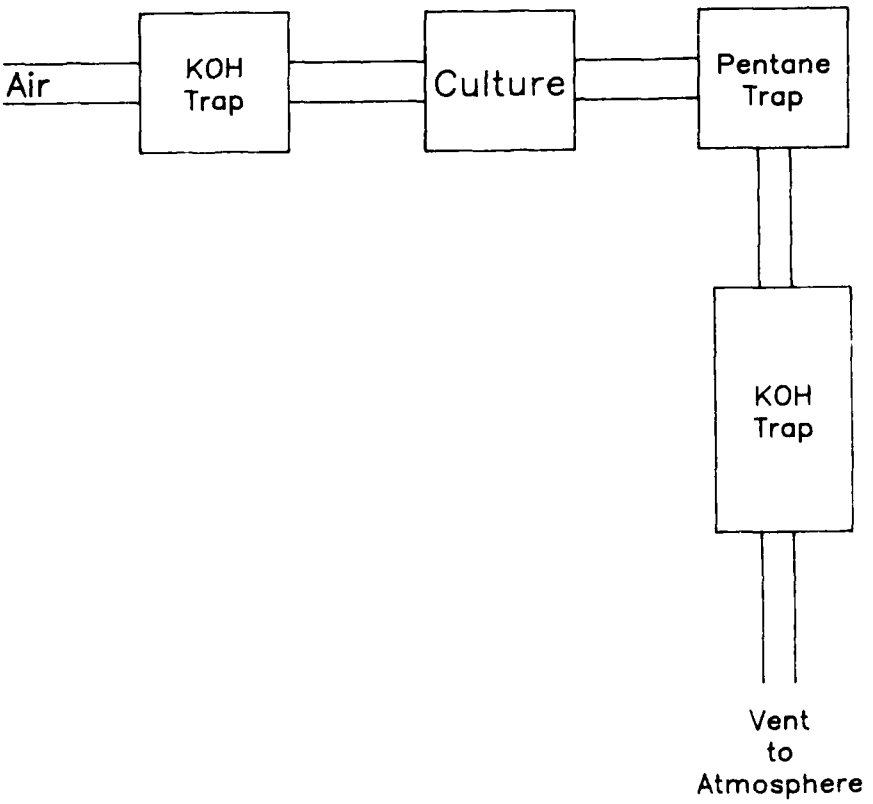

Figure 7. Radiolabel Flush Experiment Schematic

This method was used in an experiment in which radiolabeled and unlabeled TCE (4 parts label to 96 parts unlabeled TCE) was added to each of 11 flasks containing 100 mL of 1 percent glucose-basal salts medium so that each flask contained an initial TCE concentration of 100 µg/mL. All flasks were sealed with glass stoppers. Seven flasks were inoculated with the Wurtsmith 3 mixed isolate while the four remaining flasks were used as controls. Five of the inoculated cultures and three controls contained 1 N KOH in the CO_2 trap. The atmosphere in the remaining three culture flasks (one control and two inoculates) were to be flushed through the pentane and KOH solutions (10 mL each) at the end of incubation. Carbon-14 was measured both before and after the experiment in the basal salts solution and KOH CO_2 trapping solution. Also, all samples were extracted with pentane, and the pentane and aqueous layers were measured for ^{14}C activity. Carbon-14 was measured in the pentane and KOH solution from the three cultures flushed with air, after which 1 mL of the KOH solution was acidified with 1 mL of 2 N HCL and then analyzed for evidence of residual ^{14}C. Uptake of the radiolabel by the cellular material was measured at the end of the experiment. For convenience in following the movement of carbon all ^{14}C values were converted into total carbon (µgC). No special precautions were taken to ensure aerobic conditions would be maintained within the flasks. Culture viability was judged by observing the relative turbidity of the culture solutions.

A large decrease of ^{14}C activity in the basal salts media was again observed in both the control and inoculated flasks (42 percent decrease in controls versus 47 percent decrease in inoculates) during the first 24 hours (Table 12). There were no observable differences in the extraction efficiency between the control and inoculated flasks.

In the unflushed flasks (Table 12), the ^{14}C activity measured in the KOH trap indicated once again that a fraction of the radiolabel in the medium had passed into the KOH. After 24 hours, 10 percent of the original C from the inoculated broth was found in the KOH (this value was 18 percent

TABLE 12. FATE OF TCE CARBON WITH TIME

Sample	Initial	24 hours	168 hours
	Carbon (µg)		
Broth			
Controls	1790 ± 300	1070 ± 810	960 ± 70
Inoculates	1850 ± 190	970 ± 140	990 ± 10
CO_2 Trap Solution			
Controls	13 ± 6	69 ± 25	129 ± 14
Inoculates	11 ± 5	174 ± 10	198 ± 42

of the 24-hour broth), while 4 percent (7 percent of the 24-hour broth) was in the controls' KOH. The 168-hour data were 11 percent (21 percent)for the inoculates and 7 percent (13 percent) for the controls. When the KOH was extracted with pentane a larger percentage of the ^{14}C activity remained in the inoculated culture aqueous layer compared to the controls. These data again suggested that TCE was being biologically modified.

Those flasks flushed with air gave the first hard evidence that ^{14}C activity found in KOH could be $^{14}CO_2$. As observed in the cultures containing the internal KOH trap, after 1 week, the inoculated flasks contained four times more carbon in the 10 mL KOH than the control (Table 13). All of this carbon (112 percent) remained in the KOH fraction after extraction with pentane. On the other hand, 56 percent of the control's carbon remained in the KOH solution after extraction. After acidification (same method as described previously), the inoculated flask's KOH solution experienced an 86 percent reduction in the measured ^{14}C activity compared to 58 percent in the controls (Table 13).

The cultures flushed with air were incubated for an additional 2 weeks and again flushed with air through 10 mL pentane and 10 mL KOH. Inoculated samples again contained more ^{14}C in the KOH than was observed in the control (Table 13). In total, the inoculated cultures had a total of 58 µg C which could be CO_2 while the controls had 8 µg CO_2. Because the broth was not acidified, these values do not represent that portion of the CO_2 dissolved in it.

TABLE 13. APPARENT QUANTITIES OF TCE CARBON MINERALIZED TO CO_2

Sample	KOH Solution Before Acidification		KOH Solution After Acidification		C14 Loss Due to Acidification	
	Week 1	Week 3	Week 1	Week 3	Week 1	Week 3
	Carbon (µg)					
Control	12[a]	6	5	5	7	1
Inoculated	49 ± 2	22 ± 3	7 ± 1	6 ± 1	42	16

a. Controls not duplicated due to lack of flasks.

The amount of TCE carbon (based on ^{14}C) assimilated in the biomass was measured upon termination of the experiment (3 weeks). The cells were centrifuged and washed with sterile basal salts a minimum of three times. A fraction of the washed cells were measured for ^{14}C activity with the remaining cell fraction added to pentane for extraction. The nonextractable ^{14}C is assumed to be incorporated in the cellular material in a form other than TCE. Table 14 is a breakdown of the amount of radiolabel associated with the cell material from the flushed cultures and the cultures containing the CO_2 trap. The final cell density in all the cultures was $\sim 10^8$ cells/mL. In the flush cultures, 0.8 percent of the initial TCE carbon remained in the cell pellet after the washings, and of this amount only 2.7 percent could be removed by pentane extraction. For the trap cultures these quantities were 1.3 percent and 1.7 percent, respectively.

TABLE 14. OCCURRENCE OF TCE CARBON IN MICROBIAL CELLS

Sample	Total Extracted C	
	Cell Pellet	Pentane Layer
	Carbon (µg)	
KOH Flush Cultures	15 ± 5	0.4 ± 0.0
KOH Trap Cultures	24 ± 8	0.4 ± 0.1

More ^{14}C was associated with the KOH trap cultures than with cultures that were flushed with air. This could be caused by TCE losses from purging the flasks after a 1-week incubation. Measurement of ^{14}C in the pentane solution in which the purge air was bubbled showed a 48 percent loss of TCE from the basal salts solution.

The flush experiment was repeated with the Wurtsmith 3 cultures. As was previously observed, a large fraction (57 percent) of the ^{14}C contained in the KOH was removed after the addition of 2 N HCL.

The characterization studies (see Section IV) were performed on the Wurtsmith 3 isolates cultured from the radiolabel experiment inoculates. This organism (or organisms) was enriched from the original Wurtsmith Air Force Base biomass samples sent to INEL. The isolate exhibited growth in TCE/basal salts broth and TCE degradation in the presence of glucose (in both the gas chromatography and radiolabel experiments). Growth characteristics were identified in tests performed with the isolate. A final radiolabel experiment was done under optimized conditions determined from the characterization studies. The Wurtsmith 3 isolate was grown in a buffered 1 percent glucose-basal salts medium (initial pH 6) at 25°C. A second batch of ^{14}C radiolabeled TCE (New England Nuclear, Boston, Massachusetts) was obtained for use in the experiment. The chemical and radioactive purity were evaluated by gas chromatography and pentane extraction. Labeled and unlabeled TCE (4:96) were added to 100 mL of the buffered basal salts medium at an initial concentration of 100 µg/mL. Stock cultures of the Wurtsmith 3 organisms were grown in 1 percent glucose-100 µg TCE/mL basal salts medium. The cells were centrifuged, washed, and an aliquot was added to the test flasks. The control flasks were not inoculated. The cultures were then incubated for 4 days and the flushed flock atmosphere was analyzed for the presence of $^{14}CO_2$ as previously described. Data acquired in the previous growth and radiolabel experiments showed that biodegradation of TCE becomes evident during the first week of the culture incubation (in cultures containing actively growing cells). All the samples were extracted with pentane, and the pentane and aqueous layers measured for ^{14}C. This experiment evaluated the effect optimized growth had on TCE degradation.

The buffered basal salts medium in the inoculated and control flasks exhibited TCE losses of 73 and 62 percent, respectively. Only a small quantity (165 dpm) of ^{14}C was measured in the KOH through which the inoculated culture was flushed. Previous experiments using the flush procedure have all shown increased ^{14}C in the KOH solution. There were no significant difference in ^{14}C activity in the KOH solution after acid was added to the samples (165 dpm before acidification, 153 dpm after acidification). Extraction of the culture broth indicated that 99 percent of the labeled TCE could be extracted into the pentane layer. These results were in contrast to the $\sim$80 percent extractability of TCE observed in the first radiolabeled TCE batch obtained from New England Nuclear and unlabeled extraction studies. The only different parameters in this experiment from the previous studies were in the culture medium and the source of radiolabel. A buffered basal salts medium was employed to improve growth conditions. The experiment was repeated, using the original 1 percent glucose-basal salts medium to determine if the buffered medium affected TCE utilization by the cells. Carbon-14 measurements were taken after 4 days of incubation. Measurement of the KOH solution again exhibited a fraction of ^{14}C activity (222 dpm) measured from previous flush experiments. Acidification of the potassium hydroxide showed no significant decrease in labeled ^{14}C. Extraction of radiolabel from the broth approached 99 percent efficiency. A new batch of radiolabeled TCE was used in these final two experiments. The quality assurance data for the two supplies of radiolabel used in all the ^{14}C studies show a chemical purity $\geq$98.5 percent. No unknown peaks were seen in gas chromatography analysis done in our laboratory. Isolates sampled from the radiolabel experiments which exhibited possible $^{14}CO_2$ production from TCE mineralization were preserved by ultrafreezing (-70°C).

SECTION IV

CHARACTERIZATION

Characterization studies were done to identify the microorganism (or microorganisms) responsible for TCE degradation and determine optimal physiological, chemical, and nutritional growth conditions. During the enrichment and degradation studies, the cultures were streaked on basal salts agarose plates which contained TCE to obtain isolated colonies. Isolates from the Wurtsmith 3 samples were selected because these organisms exhibited the greatest capability to degrade TCE in the presence of glucose. Morphological examination of the isolates indicated that the cultures contained three similar strains. Each isolate was subjected to various physiological, nutritional, and cytological tests. Pure strains were obtained by streaking the isolates onto TCE-glucose/basal salts agarose plates and selecting isolated colonies. These colonies were used in all characterization tests. Two strains of bacteria, Pseudomonas sp. and Bacillus pulvifaciens, were obtained from the American Type Culture Collection (Rockville, Maryland) and used in the tests as positive and negative controls.

A. TAXONOMIC STUDIES

The Wurtsmith 3 bacteria were found to be gram-negative nonpigmented rods. All stains were made with log-phase cultures. Gram stains with older isolates were not definitive as these rods stained gram-variable.

The identification of the organisms was achieved using the Multitest Analytical Profile Index (API) -20E System (Analytab Products Inc., New York, N.Y.). The test procedure uses a standardized system designed for the performance of 23 biochemical tests. Interpretation of the test results enables accurate identification of gram-negative bacteria. Duplicate laboratory tests were done to verify the API results.

Oxygen requirements of the organisms were studied using the Gas Pak® (BBL Products, Baltimore, Maryland) culture system and thioglycolate growth broth. Both systems provide anaerobic growth conditions. Boiling of the thioglycolate broth drives oxygen from the medium, allowing evaluation of growth over a range of Eh potential. Growth was observed in both the Gas Pak® and thioglycolate cultures. Growth was seen throughout the thioglycolate broth (anaerobic, microaerophilic, and aerobic layers). Based on this information, the organism can be classified as a facultative anaerobe.

Three physiological tests of primary importance are the oxidase test, the catalase test, and the determination of whether sugars are oxidized or fermented. These tests, used with the bacteria's morphological characteristics, narrow the choice for determining to which major group the isolates most likely belong. All three isolates were oxidase negative, catalase positive, and produced acid and gas through carbohydrate fermentation. The other test results used for strain identification were identical for all three isolates. Table 15 outlines the bacteria's characteristics. Comparison of these results with the major keys provided by the API Profile Recognition System (Reference 18) and the Bergey's Manual of Determinative Bacteriology (Reference 19) allowed for the identification of the isolates. Only those test results important for identification of the isolates are included in Table 13.

The taxonomic studies show that the organism predominant in the biodegradation culture studies is a gram-negative straight rod. The bacterium is a motile, facultative anaerobe having both respiratory and fermentative types of metabolism. Biochemical and physiological tests indicate that the organism belongs in the genus Citrobacter. Members of the genus are grouped in the Enterobacteriaceae family. Citrobacter are commonly found in soil, water, sewage, and food. Citrobacter were the predominant organisms observed in the Wurtsmith 3 culture. Morphological examination did indicate other less predominant strains. These organisms appeared to become less viable when the Wurtsmith 3 samples were transferred into fresh growth media every 1-2 weeks.

TABLE 15. WURTSMITH 3 ISOLATE CHARACTERISTICS

Gram Stain	-
Motility	+
Oxidase Reaction	-
Catalase Reaction	+
Urease Reaction	-
Tryptophan Deaminase Reaction	-
Beta-galctosidase Reaction	+
Indole Production	-
Voges-Proskaver Test	-
Gelatin Hydrolysis	-
Utilization of:	
Citrate[a]	-
Glucose	+
Mannitol	+
Sorbitol	+
Rhamnose	+
Amygdalin	±
Arabinose	+

a. Strain was able to utilize citrate in a 0.1 percent (w/v) concentration.

B. GROWTH STUDIES

The growth rate of the mix culture bacteria in a basal salts medium (supplemented with 1 percent glucose and 100 µg/mL TEC) was determined under a variety of conditions. Parameters examined include pH, temperature, nitrogen, and various organic nutrients. Evidence of growth of the cultures was obtained by absorbence measurements with a Bausch and Lomb Spectronic-20 spectrophotometer and viable cell counts on spread plates of serially diluted samples. The linearity of the calibration curve for the spectrophotometer measurements (optical density) and the relationship of optical density to spread plate counts were evaluated in preliminary growth experiments. Absorbence measurements and spread plate counts were made throughout logarithmic growth, and data were plotted on a graph (absorbence measurements versus spread plate counts). A correlation coefficient >0.98 was obtained between the two growth measurements.

1. pH

The first growth parameter investigated was pH. Generation times were calculated from absorbance data obtained on cultures grown in 1 percent (w/v) glucose-basal salts medium containing 100 µg/ml TCE at initial pH values ranging from 4.0 to 9.0. However, pH control was very difficult because of precipitate formation and the low buffering capacity of the medium. A modified basal salts medium having a buffering capacity over the examined pH ranges was used for growth measurements. It was found that use of this buffered medium supported a denser concentration of cells than the 1 percent glucose-basal salts medium. A five-to-tenfold increase in cell numbers was observed at the end of logarithmic growth.

Cell growth did occur in just the buffer solution (without a supplemental carbon source) after a lag period of 1-2 days because of the small amount of citrate ($\leq$0.04 percent) present in the medium. This lag period was not observed when glucose was present in the medium.

The results of the growth experiments determining the range of pH tolerance are shown in Figure 8. No growth was observed at pH 4 or 9 after 48 hours. However, cells held at pH 4 after this period did exhibit slight growth in the cultures. The optimum pH for growth was at pH values ranging from 6 to 7.

2. Temperature

The influence of temperature on growth was examined to determine the optimal temperature for growth. Cultures were grown at 5, 15, 25, and 35°C. The strain was capable of growth at all temperatures except 5°C, with the optimum temperature for growth located at 35°C (Table 16).

3. Nitrogen

Another growth factor evaluated was nitrogen content. The form and concentration of nitrogen can be growth-limiting. Individual nitrogen sources were added to the buffered basal-salts medium (pH 6) and the

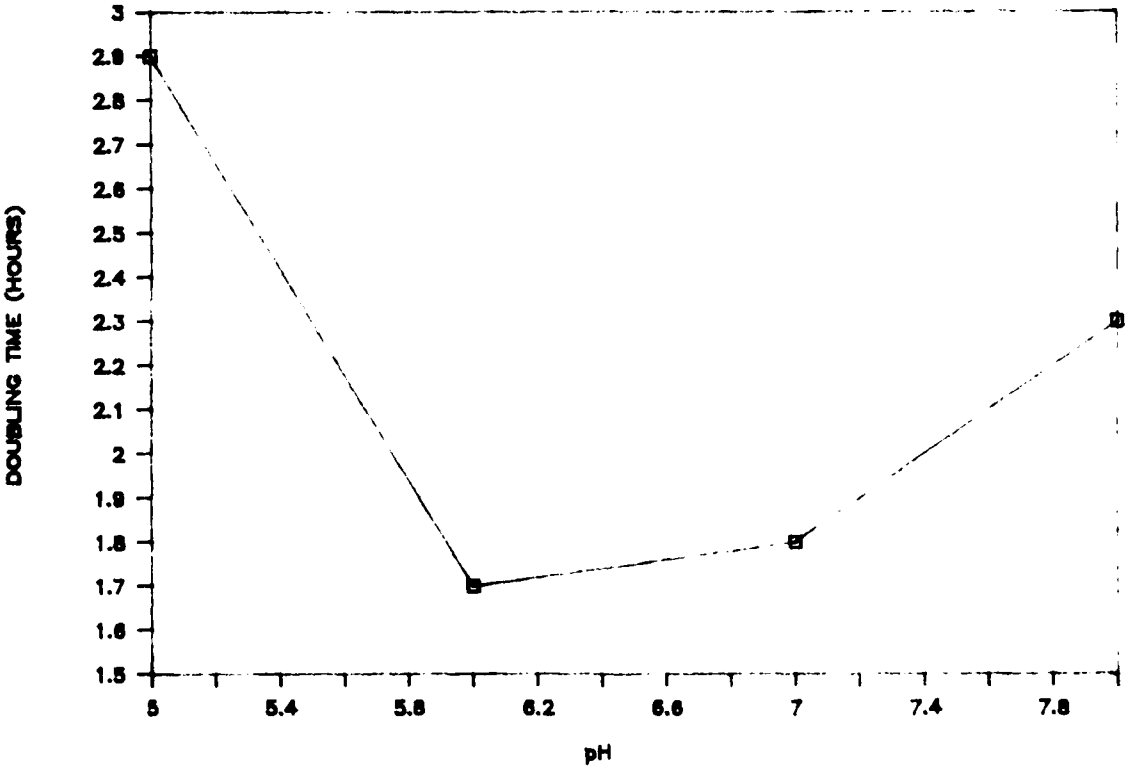

Figure 8. Effect of pH on Doubling Time

TABLE 16. GROWTH RATE IN BUFFERED BASAL SALTS MEDIUM (pH 6.0) AT DIFFERENT TEMPERATURES

Temperature (°C)	Doubling Time (hours)
5	No growth
15	3.3
25	1.7
35	1.6

cultures examined for growth. The initial evaluation examined different compounds of nitrogen and their effect on cell growth. Peptone, L-alanine, potassium nitrate, and ammonium sulfate were tested at 0.1 percent (w/v). Growth was obtained on all nitrogen sources except potassium nitrate and control cultures (1 percent glucose-buffered basal salts medium containing no nitrogen). The maximum cell density was observed in the peptone/basal salts medium. Peptone contains nitrogen in a form readily available for bacterial growth requirements. It has a high peptone and amino acid content and only a negligible quantity of more complex nitrogeneous constituents. A high biomass concentration can be expected using the hydrolyzate.

Ammonium sulfate also produced good bacterial growth. The compound was used as a nitrogen source in the biodegradation experiments. The effect of ammonium sulfate concentration on growth was then evaluated by adding the compound to cultures at a final concentration of 0.01, 0.1, and 1.0 percent (w/v). The effect of ammonium sulfate on the cell doubling time is given in Table 17.

4. Carbon Utilization

Individual carbon sources were added to the basal salts medium. The buffered medium was not used in these tests to ensure that possible citrate utilization did not interfere with the test results. The carbon sources tested included those which are relatively economical for use in large-scale biological operations, i.e., bioreactors. These compounds include acetate, ethanol, methanol, and citrate. All flasks were inoculated

TABLE 17. AMMONIUM SULFATE UTILIZATION

$(NH_4)_2SO_4$ Concentration[a] (µg/mL)	Doubling (hours)
0.1	1.3
1.0	1.7
10.0	1.8

a. Incubation temperature 25°C, pH 6.

from cultures grown in a 1 percent glucose/100 µg TCE/ml basal salts medium. Carbohydrate utilization was evaluated in the taxonomic studies. Two halogenated organic compounds, 1,2-dichloroethane and 1,2-dibromoethane, were added to the basal salts at a concentration of 100 µg/mL. These compounds are similar to TCE in that they are two-carbon halogenated organics, have a high vapor pressure, and are only slightly soluble in water. All culture flasks were incubated at 25°C for a minimum of a week.

Results in the carbon and nitrogen source studies are summarized in Table 18. Growth was not observed in cultures with acetate, methanol, ethanol, or dichloro- and dibromoethane as the sole added carbon source after a 1-week incubation time. The doubling time, using glucose as the carbon source, was 1.7 hours. The isolate could only utilize citrate in concentrations not exceeding 8 µg/mL. The organism's doubling time for citrate varied between 1.7 to 2.0 hours. The addition of 10 and 100 µg/ml TCE to the 1 percent glucose-basal salts medium did not affect the velocity of growth by the isolate. TCE at an added concentration of 100 µg/mL caused a 48-72 hour lag phase before growth was noted. After this initial lag phase growth, velocity was not affected. This showed that the organism adapted readily to various concentrations of TCE.

TABLE 18. UTILIZATION OF VARIOUS CARBON AND NITROGEN SOURCES

Medium	Growth
Carbon Source	
Acetate	-
Ethanol	-
Methanol	-
Glucose	+
Citrate	+
1,2-Dichloroethane	-
1,2-Dibromoethane	-
Mannitol	+
Inositol	-
Sorbitol	+
Rhamnose	+
Sucrose	-
Melibiose	+
Amygdalin	±
Arabinose	+
Nitrogen source[a]	
Potassium nitrate	-
Ammonium Sulfate	+
Peptone	+
L-alanine	+

a. Nitrogen source added to 1 percent glucose-buffered basal salts.

SECTION V

CONCLUSIONS

The ability of a microbial population to degrade TCE was not substantiated. However, batch cultures containing TCE as the sole apparent organic carbon source in concentrations up to 1000 µg/mL did support microbial activity. In the presence of the glucose (1 percent w/v) and TCE (radiolabeled and unlabeled TCE) degradation of TCE apparently took place as evidenced by the assimilation of ^{14}C in cellular material and the production of $^{14}CO_2$. Extraction of the CO_2 trap solution and the cellular material with pentane indicate that the ^{14}C is present in a polar form, i.e., nonextractable. This information strongly suggests that the nonpolar TCE molecule was transformed into a polar form by the cultures, i.e., microbial metabolism. Subsequent experiments designed to achieve increased TCE utilization by the microorganism characterized as belonging in the genus Citrobacter exhibited no degradation of TCE. Culture conditions included using both optimal growth conditions (pH = 6, citrate buffered basal salt, optimal nitrogen concentration) and the original enrichment media outlined in Table 2. The strain (or strains) responsible for the apparent TCE degradation could have lost viability during subculturing. Frequent subculturing of microorganisms can result in contamination, selection of variants, mutants, or possible loss of culture (Reference 13).

Earlier cultures did exhibit morphological variety compared to cultures examined in the latter stages of the investigation. This increased homogeneity was assumed to be due to the selection of an organism (or organisms) able to degrade TCE. All cultures were TCE tolerant, i.e., growth was observed in TCE concentration up to 1000 µg/mL.

SECTION VI

RECOMMENDATIONS

Several of the studies performed in the investigation indicated apparent TCE degradation. These results were not duplicated in studies designed to increase this apparent metabolism of TCE. The original samples which have been preserved should be reviewed and evaluated for the capability to degrade TCE. These samples appeared to contain a heterogeneous mixture of microorganisms compared to later cultures.

Any further work should evaluate TCE biodegradation using gas chromatography, radiolabel, and respirometry methods, Gas chromatography headspace analysis is one technique recommended to differentiate abiotic (volatilization) or biotic (biodegradation) loss of TCE.

Carbon flow studies using radiolabel TCE should be repeated. Cell fractionation should be performed on the washed cell pellets to determine where ^{14}C is assimilated by the cell, i.e., in the cell membrane, cell protein etc.

Since the cultures were aerobic, oxygen uptake by the cultures should be examined. Oxygen demand measurements (respirometry) are an excellent tool for examining the metabolic process. Respirometry measurements should be included in any further work as an indicator of metabolic activity in the TCE cultures. Necessary measurements include evaluating the effect of TCE concentration and consubstrate use on respiration. Increased respiration (compared to the endogeneous demand) would indicate TCE utilization.

The experimental design for all studies should include a sufficient number of replicates to enable statistical analysis.

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